DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR ENUMERATION OF FECAL INDICATORS AND EMERGING CHEMICAL CONTAMINANTS IN BIOSOLIDS

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Science Questions

Multi-Year Plan Science Question: What improved analytical techniques can be developed to adequately determine pathogen and priority toxic chemicals in or released from biosolids?

Research Question: What techniques must be employed to ensure that analytical methods adequately determine the density of indicator organisms, specific pathogens, and concentrations of pharmaceuticals and personal care products (PPCP) in biosolids?

How Research Addresses the Water Quality MYP Goals

This research is designed to provide clients with standard protocols which can accurately and precisely quantify specific pollutants in wastewater sludges and biosolids. With respect to microbial analysis, regulated indicators and specific pathogens are 50% completed, while work on PPCP analytes is just getting started. Ultimately this research will provide the approaches, methods and tools to assess exposure and reduce the human health risks from specific biosolid contaminants.

Research Objectives

Land Application of Biosolids

Solid particles from sedimentation and biological processes used in the treatment of municipal wastewaters are commonly referred to as sludge. Sludges which have been appropriately treated are called biosolids. In 1993 the U.S.EPA published standards for the use or disposal of sewage sludge (40CFR503) which specify treatment and monitoring requirements dependant upon its disposition. Under these standards, a Class B biosolid may be land applied for agricultural purposes if it received appropriate treatment or if it has fewer than two million fecal coliform per gram on a dry weight basis. Similarly, Class A biosolids must have received appropriate treatment and must not contain greater than 1000 fecal coliform per gram on a dry weight basis or three salmonellae per gram dry weight. The standards also allow for monitoring specific pathogens (enteric viruses and viable helminth ova) prior to land application in lieu of using an approved treatment. The analytical techniques specified under the standards and used for monitoring indicator organisms and pathogens in biosolids were obtained from protocols developed for analysis of water and wastewater which were adapted for detection and enumeration of such organisms in biosolids. However, microorganisms are known to sorb and bind to solid particles. Such binding can affect detection and quantification of organisms which leads to observed differences in microbial densities when heterogeneous solids such as biosolids are assayed.
Similarly, the use of pharmaceuticals and personal care products (PPCP) throughout the U.S. has increased in recent years. Many of these substances are known to sorb to solid particles and may concentrate in biosolids. The extent to which these chemicals concentrate and persist in biosolids when applied to land remains unknown because analytical methods have not been developed and/or evaluated.
The objective of this work is to develop standard protocols for detection and enumeration of indicator organisms (fecal coliform) in biosolids and to assess the within and between lab variability of the methods. Additionally, this work is also designed to identify the most appropriate methods for analysis of specific pathogenic microorganisms in biosolids, develop standardized protocols and verify the accuracy of such methods. Also, this work is designed to develop appropriate methods for analysis of certain PPCPs in biosolids for use in future survey work in order to determine the fate of these substances following biosolids land application.

Research Methods & Collaboration

Fecal coliform analysis

Growth in Lauryl Tryptose broth

and Confirmation on EC broth

The analytical method currently approved by EPA for fecal coliform analysis of biosolids is *Standard Methods* 9221E, which is designed for use in water and wastewater.
EPA Methods 1680 and 1681 are adaptations of *Standard Methods* 9221E for use in biosolids. Both use a multiple fermentation tube approach for estimating the MPN of fecal coliforms in a sample.
LTB/EC: A presumptive step using lauryl tryptose broth (LTB) plus a confirmation step using EC broth. (EC broth must not be used for direct fecal coliform isolation from a biosolid sample because prior enrichment is required in LTB medium for optimum recovery of fecal coliforms).
A-1: A direct, single step test using A-1 medium.
A two laboratory single sample source methods comparison study was conducted to select the best available method for detection and enumeration of Salmonella sp. in biosolids. Results of this work demonstrated that enrichment followed by selection using a modified semi-solid Rappaport-Vasiliades (MSRV) medium yielded higher recovery of organisms in Salmonella spiked biosolid derived compost. Based upon these results the protocol for use of this technique was further developed for multilab comparison testing of Class A and B biosolids.

Collaborations:
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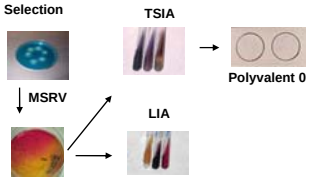
Sanwat Chaudhuri and Devon Cole - Utah Department of Health
Nancy Hall and Cathy Lord - University of Iowa Hygienic Laboratory
John Standridge and Linda Peterson - Wisconsin State Lab of Hygiene
BioTechnology Frontiers

Bioball used for spiking biosolids

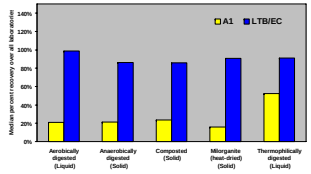
***Salmonella* sp. Selection and biochemical confirmation**



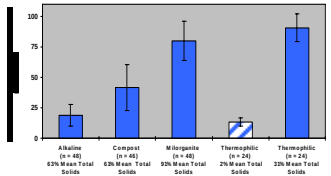
Research Results

Two methods for analyzing fecal coliform and one method for detection and enumeration of Salmonella sp. in biosolids were evalutated by 12 laboratories. Results showed that recovery of spiked *Escherichia coli* (a member of the fecal coliform group) was better using the LTB/EC method. However, analysis of split samples obtained from municipal wastewater treatment facilities showed no significant difference in response regardless of which fecal coliform method was used.
A similar approach was taken for validation of the MSRV method for analysis of *Salmonella sp*. in biosolids. A lyophilized culture containing a known density of *S. typhimurium* was used to spike five types of Class A biosolids. Recovery of sample spikes was acceptable for all samples except for the sample which had undergone thermophilic treatment. The low recovery from this matrix suggests toxicity and indicates that it may be inappropriate to use this method for analysis on thermophilically treated biosolids.

Median Percent Recovery EPA Methods 1680 and 1681



EPA Method 1682: *Salmonella* Recovery Based on BioBall™ Lot Mean Value (43.4 *Salmonella* per BioBall™)



Research Conclusions & Future Directions

Standardized and validated methods for fecal coliform and *Salmonella* sp. in biosolids have been developed based upon the analytical protocols identified in the current federal regulation. Recovery rates for spiked sample was found to be high. Within lab and between lab recoveries were considerable, yet results for unspiked samples showed little within and between lab variation.

Further development of methods to accurately determine the presence and number of pathogens in biosolids and sludges is needed. The standardized and validated methods for fecal coliform and Salmonella sp. demonstrate a high degree of variability in the recovery of spiked organisms which suggests that consistent results using such methods is difficult.
Future work includes working with the University of Cincinnati to develop improved techniques for elutriation, detection, and identification of enteric viruses in biosolids. The improved techniques will be able to detect many enteric virus types that are not detected by the current analytical method. Upon completion of this effort, a standardized protocol will be developed and, if funding is available multilab validation testing will be conducted similar to the approach used for fecal coliform and *Salmonella*. EPA also has plans to begin research to optimize the method for isolating and identifying viable ova of the parasitic helminth (worm), *Ascaris lumbricoides* in biosolids.

PPCP's are part of waste streams entering publicly owned treatment works and may concentrate in sludges. EPA is currently developing methods for the detection and quantification of specific PPCPs in biosolids. Using a previously developed analytical method for synthetic musk compounds, extraction techniques were carried out to determine the most efficient extraction method. A pressurized liquid extraction technique (PLE) was adopted. The objective was to develop a higher percentage recovery (close to 100 %) of synthetic musk compounds in Class A biosolids. Although the method has been developed, details of the analytical work have not been presented. Method development for recovery and detection of fluoroquinolone and macrolide antibiotics are underway. Both sonication, liquid-liquid, and accelerated solvent extraction (ASE) are being investigated as possible extraction techniques. High performance liquid chromatography (HPLC) with fluorescence and HPLC with mass spectroscopy (MS) are being investigated as detection techniques.

Interactions with Customers

Key clients:
EPA's Office of Water worked collaboratively with ORD to develop and implement the fecal coliform and *Salmonella* sp. methods multilab validation studies.
EPA Regional Offices provided insight and comments on approach used for compliance testing.
State Agencies responsible for compliance monitoring are informed of progress and participate in validation work.
Technology Developers are informed of method development work and the potential impact new/revised methods may have on their technologies.
State and private laboratories have participated in validation studies and contributed to standardization of methods
Wastewater treatment facilities have provided process samples for method development and validation studies.

How Research Contributes to Outcomes

OW intends to publish the fecal coliform and *Salmonella* methods in 40 CFR Part 136 as EPA approved methods for analysis of biosolids. Following publication, these methods will become the standard protocols for compliance monitoring throughout the US. Similar uses are expected for the virus and helminth ova analytical techniques. In addition, the microbial methods are used by stakeholders and EPA's Pathogen Equivalency Committee (PEC) to evaluate the treatment effectiveness of alternative biosolids disinfection techniques. Improved methods are also needed to conduct national exposure studies on pathogens in biosolids. Knowledge of available measurement methods for PPCPs will help OW decide whether to include certain PPCPs in national surveys to obtain source concentration data. Finally, the availability of more technically sound methods for the analysis of pollutants in biosolids is expected to result in improved treatment, thereby reducing the risk of human health or ecological impacts from their land application.